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SUBSTRATE-INDUCED H_2O_2 PRODUCTION IN MYCELIA FROM THE LIGNIN-DEGRADING FUNGUS PHANEROCHAETE CHRYSOSPORIUM

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Broken and subsequently washed mycelia from the lignin-degrading fungus Phanerochaete chrysosporium generated increased levels of H_2O_2 when exposed to a lignocellulosic substrate (ground wheat straw) or substrate analogue (Poly B-411). H_2O_2 production was observed only in cultures grown on limiting nitrogen, a condition known to induce the lignin-degrading activity of this organism. H_2O_2 production was observed in the pH range of 4.5 to 8.4, but the highest levels of H_2O_2 were generated around pH 6. The mycelia also exhibited catalase activity, which was 5- to 10-fold higher in nitrogen-limited cultures.

The biochemistry of lignin degradation by organisms such as the Basidiomycete (white rot) fungi is poorly understood. Studies with Phanerochaete chrysosporium Burds., the best characterized of these organisms, have indicated that lignin degradation is probably a secondary metabolic event, ocurring after primary growth has slowed or stopped (1, 2). Commonly, limiting nitrogen is employed to stress the organism and induce lignolytic activity (1, 3). Initial lignin breakdown is an oxidative, extracellular process (4-7). It has been postulated that excreted oxidants such as H_2O_2 or $^{\cdot}OH$ are involved in the initial depolymerization reactions (6, 7). Indeed, both H_2O_2 and $^{\cdot}OH$ have been identified in the media and in preparations of P. chrysosporium cultures (6, 7), and the level of these oxidants appears to be correlated temporally with the amount of lignolytic activity. In our laboratory, the highest concentration

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

[2]Poly B-411 is a water-soluble, blue dye comprised of anthraquinone residues covalently linked to a polyvinyl backbone (16). Strong evidence has been provided which indicates that the lignin degrading system of P. chrysosporium also decolorizes Poly B-411 (17).

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of H_2O_2 found in the media of lignolytic P. chrysosporium cultures was low ($<10^{-4}$M). By itself, this level of H_2O_2 did not significantly react with lignin even in the presence of metal ions, which can catalyze a Fenton-type reaction to produce $^{\cdot}OH$ (unpublished data). The $^{\cdot}OH$ radical is an unlikely candidate for an excreted oxidant because of its extremely high reactivity with virtually all organic molecules (8). Furthermore, as a result of this high reactivity and low specificity, the diffusional lifetime of $^{\cdot}OH$ in complex solutions is extremely short (L. K. Patterson, personal communication). It is possible that relatively high local levels of H_2O_2 (or $^{\cdot}OH$) could be produced enzymatically in close proximity to lignin, so that problems arising from short diffusional lifetimes and low bulk concentrations are effectively avoided. Such a mechanism requires a close physical association between the oxidant-producing enzyme system and the lignin substrate.

An attractive alternative has recently been suggested by Tien and Kirk (9). These authors purified an enzyme from the media of lignolytic P. chrysosporium cultures that, in the presence of H_2O_2, partially degraded lignin model compounds and solvent-extracted spruce and birch lignins. Based on this finding, Tien and Kirk concluded that at least one key reaction in lignin biodegradation is catalyzed by an extracellular H_2O_2-requiring enzyme.

In either model it would be advantageous for P. chrysosporium to possess a system capable of recognizing the presence of lignin in its environment. This would allow the organism to activate and/or excrete the lignin-oxidizing system only when substrate is present, thereby conserving metabolic energy. In this communication we report a necessary characteristic of a lignin recognition system in P. chrysosporium; namely, an increased production of extracellular H_2O_2 in response to the presence of a lignocellulosic substrate or a lignin analogue.

MATERIALS AND METHODS

Phanerochaete chrysosporium Burds. was grown as previously described (10) following the basic method of Kirk et al (3). Low-nitrogen medium contained 0.6 mM NH_4NO_3 and 0.6 mM asparagine. High-nitrogen medium contained 6 mM NH_4NO_3 and 6 mM asparagine. Cultures were grown in a volume of 500 ml in 3 liter Fernbach flasks without shaking. After 10 days at 37°C, a large mat

of entangled mycelia was present in the flask. The mycelial mat (about 15 g wet weight) was harvested by filtration and washed twice with 300 ml of 20 mM K_2HPO_4 (pH 6.2). Size and physical characteristics made the mycelial mat unsuitable for the type of experiments conducted in this study. Therefore, it was broken into smaller pieces and slurried in a Waring Blendor (3 repetitions of 10 sec) containing 50 ml of 0.4 M KCl, 20 mM Na_2HPO_4 (pH 6.2). The resulting suspension was centrifuged at 20,000 X g for 5 min. The pelleted pieces of mycelial mat were gently resuspended in 50 ml of fresh 0.4 M KCl, 20 mM Na_2HPO_4 (pH 6.2) and centrifuged again. The washed pellet was resuspended in fresh medium (0.4 M KCl, 20 mM Na_2HPO_4 at pH 6.2, unless otherwise stated) to a final concentration of ca. 2 mg protein·ml^{-1}. The final mycelial preparation contained small pieces (<3 mm diameter) of the original mycelial mat, each composed of numerous intertwined segments of apparently intact mycelia.

Changes in O_2 concentration in the mycelial preparation were monitored polarographically in a thermostatted (30°C), water-jacketed cell (Gilson Medical Electronics) using a Clark electrode connected to a strip chart recorder. Protein concentration of the mycelial preparation was determined by the method of Lowry et al (11), after disruption of the mycelia with a Branson model S125 sonifier. Wheat straw, obtained locally, was ground to pass a 2-mm screen and washed repeatedly with distilled water. Catalase and Poly B-411 were purchased from Sigma. All other chemicals were reagent grade.

RESULTS

Catalase-mediated H_2O_2 decomposition has been described for numerous biological systems (12-15). Because the presence of catalase activity associated with the washed P. chrysosporium mycelial preparation would reduce apparent H_2O_2 production, the catalase activity of our preparation was assayed by adding exogeneous H_2O_2 and monitoring subsequent O_2 evolution. Considerable catalase activity was exhibited by mycelia from both high and low nitrogen cultures. In both cases the activity could be greatly inhibited (>90%) by 200 μM NaN_3 (Fig. 1), although mycelia grown on low nitrogen always exhibited 5-10 times higher catalase activity than mycelia grown on high nitrogen.

The level of H_2O_2 in extracts from nitrogen-limited cultures of P. chrysosporium has been shown to increase dramatically at the same time the cultures develop the ability to convert ^{14}C-lignin into $^{14}CO_2$ (6). This correlation suggests that H_2O_2 is involved in extracellular lignin degradation. The possibility of H_2O_2 involvement is further supported by the data presented in Figure 2, which shows the rate of extracellular H_2O_2 production by washed mycelia from low-nitrogen cultures in the presence of various substrates. With no substrate present, the mycelia produced H_2O_2 slowly for a period of several hours. If ground wheat straw was present, the amount of H_2O_2 produced was increased more than twofold. The water-soluble lignin analogue, Poly

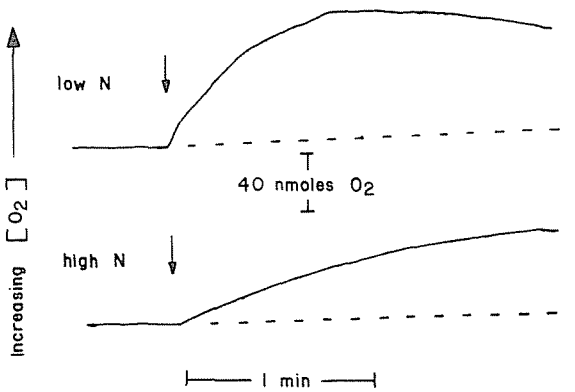

Figure 1. Endogenous catalase activity in P. chrysosporium mycelia. Mycelial preparations were from low-nitrogen (upper trace) and high-nitrogen (lower trace) cultures. Catalase activity was assayed in 1.3 ml of the respective preparations by monitoring O_2 evolution after addition of H_2O_2 to a final concentration of 1.9×10^{-4} M (designated by the arrows). The dashed lines indicate the response observed in the presence of 2×10^{-4} M NaN_3. Protein concentrations for the low-nitrogen and high-nitrogen mycelial preparations were 1.6 mg·ml^{-1} and 2.1 mg·ml^{-1}, respectively. Standardization of changes in O_2 concentration was accomplished by addition of 300 µg of exogenous catalase and known quantities of H_2O_2.

B-411[2], caused an even larger increase in the amount of extracellular H_2O_2. The production of extracellular H_2O_2 was observed only in mycelia from cultures of P. chrysosporium grown with limiting nitrogen. Mycelia from cultures grown

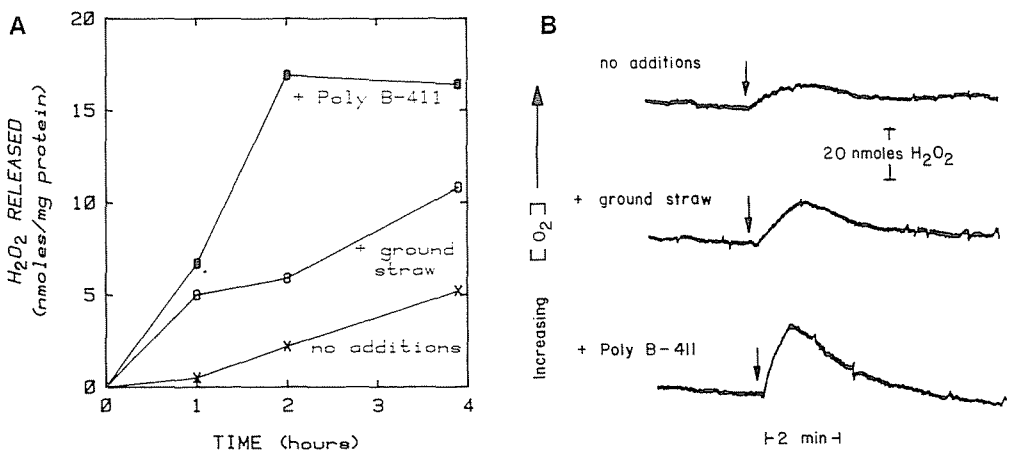

Figure 2. Extracellular H_2O_2 production stimulated by ground straw or Poly B-411. A mycelial preparation (1.6 mg protein·ml^{-1}) was prepared from a low-nitrogen culture and divided into three reaction vessels, each containing 2×10^{-4} M NaN_3. Aliquots (1.3 ml) were taken at the designated time intervals and H_2O_2 content was assayed by overwhelming the system with exogenous catalase (300 µg) and monitoring subsequent O_2 evolution. Addition of a known quantity of H_2O_2 was used for standardization. Panel (A): Additions to the reaction vessels were none (X), 5 mg ground straw·ml^{-1} (O), or 0.2 mg Poly B-411·ml^{-1} (●). Panel (B): Raw data after 4 hours incubation. The arrows designate the addition of catalase.

in high-nitrogen medium did not produce measurable amounts of H_2O_2 after 4 hr in either the presence or absence of ground straw or Poly B-411 (not shown).

The effect of medium pH on H_2O_2 production by the mycelial preparation in the presence and absence of Poly B-411 is shown in Figure 3. H_2O_2 production was observed in the pH range of 4.5 to 8.4, and Poly B-411 stimulated the production throughout this range. Interestingly, maximal H_2O_2 production occurred between pH 5.5 and pH 6.5. This was a somewhat unexpected result, because it was reported earlier that the optimum pH for lignin degradation is 4.5 (3). Throughout the pH range examined in Figure 3, endogenous catalase activity was found to be inhibited >90% by 200 μM azide (not shown). Therefore, it is unlikely that this pH profile was skewed by residual catalase activity.

DISCUSSION

Evidence is accumulating which indicates that H_2O_2 may play an important role in the mechanism of fungal lignin degradation (5-7, 9, 18). The observation that a lignocellulosic substrate or a lignin analogue accelerates the production of H_2O_2 by P. chrysosporium mycelia (Figs. 2 and 3) further supports this hypothesis. The higher levels of catalase activity associated with lignin-

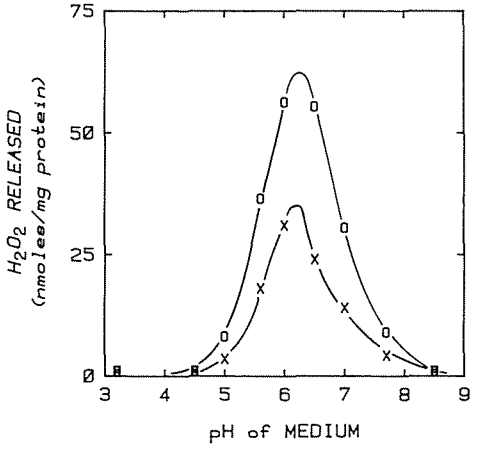

Figure 3. Effect of medium pH on H_2O_2 production. A mycelial preparation (1.4 mg protein·ml^{-1}) was suspended in 0.4 M KCl, 10 mM Na_2HPO_4, 10 mM 2,2-dimethylsuccinate, 200 μM NaN_3 and was divided into reaction vessels. The pH of each suspension was then adjusted with NaOH or HCl. The symbols designate no additions (X) or the addition of 0.2 mg Poly B-411·ml^{-1} (O) to the reaction vessels. H_2O_2 content was assayed as in Figure 2 after 4 hours of incubation.

degrading cultures (Fig. 1) also supports this conclusion, since increased catalase activity would probably be required to protect intracellular components from the higher levels of H_2O_2 in the lignin-degrading cultures. Furthermore, Tien and Kirk (9) have purified an extracellular lignin-degrading enzyme that requires H_2O_2 for activity. It is possible that all or part of the catalase activity observed in our mycelial preparations (Fig. 1) may be attributable to the same enzyme, especially since many peroxidases are known to exhibit substantial catalase activity at higher H_2O_2 concentrations (19). If this is indeed the case, we would predict the enzyme isolated by Tien and Kirk to be azide sensitive.

The observed pH optimum for H_2O_2 production (~pH 6, Fig. 3) does not correlate well with the previously reported pH optimum for P. chrysosporium lignin degradation (~pH 4.5, Ref. 3). However, the pH optimum for lignin degradation was determined by measuring the production of $^{14}CO_2$ derived from ^{14}C-lignin. Numerous metabolic steps are involved in the conversion of lignin into CO_2. At least some of these steps probably occur extracellularly. The pH optimum of 4.5 as determined from measurements of $^{14}CO_2$ evolution must therefore represent an average of the effects of pH on the many constituent reactions, or be the effect of pH on a rate-limiting metabolic step in the overall reaction.

Demonstration of lignocellulosic-induced H_2O_2 production by P. chrysosporium mycelia suggests that this organism possesses a mechanism that responds to lignin in its environment. Two possible mechanisms for lignin recognition can be postulated. The first mechanism would utilize a receptor protein located on the surface of the mycelia. Because of the complex, three-dimensional structure and almost random arrangement of the substituted phenylpropane units in lignin (20), it seems likely that a lignin receptor would be rather nonspecific, perhaps recognizing a more general, substituted aromatic moiety. This relatively low specificity may explain why Poly B-411 is apparently degraded by the P. chrysosporium lignin degradation system (17). The second possible mechanism involves the recognition of a lignin breakdown product generated by an "idle

level" of excreted H_2O_2. Binding of this product, either extracellularly or intracellularly, could then induce accelerated H_2O_2 production. Additional studies are underway to further explore the role of the mycelial cell surface in the mechanism of lignin biodegradation, and to elucidate the metabolic source of extracellular H_2O_2.

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